ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1995, p. 1596–1598 Vol. 39, No. 7
0066-4804/95/$04.00+0

Susceptibility of *Acanthamoeba castellanii* to Contact Lens Disinfecting Solutions

STEFANIA ZANETTI,[1]* PIER LUIGI FIORI,[1] ANTONIO PINNA,[2] STEFANIA USAI,[1] FRANCESCO CARTA,[2] AND GIOVANNI FADDA[1]

Institute of Microbiology and Virology[1] and Institute of Ophthalmology,[2] University of Sassari, 07100 Sassari, Italy

Received 28 November 1994/Returned for modification 20 March 1995/Accepted 20 April 1995

A corneal isolate of *Acanthamoeba castellanii* was exposed to commercial contact lens disinfecting solutions containing hydrogen peroxide, benzalkonium chloride, polyaminopropyl biguanide, polyquaternium 1, and chlorhexidine-thimerosal. The minimum trophozoite amebicidal concentration and exposure times required to kill trophozoites and cysts were determined. Solutions containing hydrogen peroxide or chlorhexidine-thimerosal were active against both trophozoites and cysts. The benzalkonium chloride-based solution was effective only against trophozoites. Solutions containing polyaminopropyl biguanide or polyquaternium 1 were completely ineffective. The need for adequate exposure times must be stressed.

Acanthamoebae are small, ubiquitous, free-living protozoa which can exist in two forms: the motile trophozoite and the double-walled cyst (20). In the encysted state they are protected from unfavorable environmental conditions and are resistant to extremes of temperature (3, 9), dessication (10), and several antimicrobial agents (16). *Acanthamoeba* keratitis is an increasingly frequent and potentially blinding infection associated with contact lens wear (14, 15). The association between this infective keratitis and the use of contact lenses is now firmly established (4, 14). The predisposing conditions most commonly associated with the development of *Acanthamoeba* keratitis in contact lens wearers include a minor corneal trauma, exposure to contaminated water (well water, hot-tub water, and domestic tap water [6, 19]), and the use of homemade saline (14, 15). Because the cyst form of *Acanthamoeba* organisms is resistant to most antimicrobial agents at concentrations achievable in the cornea and tolerated by the ocular surface, treatment is exceedingly difficult. Prolonged medical therapy with antifungal agents or propamidine isethionate may yield a cure (1) or control the disease sufficiently to allow corneal transplantation a chance of success (5). Given the frequent failures of medical and surgical treatment, disinfecting solutions effective at killing *Acanthamoeba* organisms are important in preventing corneal infection. This study was undertaken to try to gain insight into how commercial contact lens disinfecting solutions affect trophozoites and cysts of a clinical isolate of *Acanthamoeba castellanii.*

A strain of *A. castellanii* originally isolated from the corneal ulcer of a soft contact lens wearer was obtained from the Institute of Microbiology of the University of Ancona, Ancona, Italy. Stocks of *Acanthamoeba* cysts maintained at −70°C were slowly thawed and plated on nonnutrient agar seeded with a lawn of *Escherichia coli* (ATCC 35218) (NNA-*E. coli*) for 1 day to obtain motile trophozoites. Axenically cultured organisms were prepared by transfer of the trophozoites to PYG medium (7) containing penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml) and then subcultured to antibiotic-free PYG medium; organisms were grown in tissue culture flasks (25 cm^2). Suspensions of *Acanthamoeba* trophozoites were obtained from 1- or 2-day subcultures at 37°C. Suspensions of *Acanthamoeba* cysts were obtained from 4-day subcultures of trophozoites starved in phosphate-buffered saline (PBS) at 27°C; at that time, fewer than 95% of the cells were encysted, as determined by trypan blue (0.2%) staining and phase-contrast microscope examination.

Six contact lens disinfecting solutions purchased at local retail stores were tested. Their active ingredients were hydrogen peroxide (30,000 μg/ml) (two different brands), benzalkonium chloride (40 μg/ml), polyaminopropyl biguanide (PABP) (0.5 μg/ml), polyquaternium 1 (11 μg/ml), and chlorhexidine (150 μg/ml)-thimerosal (50 μg/ml). The disinfection times recommended by the manufacturers were 30 min, 6 h, overnight, 4 h, 4 h, and 6 h, respectively.

Logarithmic-phase cultures of trophic amebas were washed twice in PBS and resuspended at a concentration of 3×10^5/ml in PYG; the suspension was distributed in 96-well plates (100 μl per well). Serial dilutions of each disinfecting solution (undiluted, 1:5, 1:50, and 1:500) in PYG were performed, and 100 μl of each dilution was added to the wells containing trophozoites, giving final dilutions of 1:2, 1:10, 1:100, and 1:1,000, respectively. Control wells received 100 μl of PYG in place of disinfecting solution. The plates were sealed with clear adhesive film and incubated at 37°C. After 24 h, the wells were inspected with a phase-contrast microscope. By comparison of the appearance of the trophozoites in the test wells to those in

TABLE 1. MTACs of six disinfecting solutions required to kill *A. castellanii* trophozoites in vitro in 24 h

Solution (concn [μg/ml])	Result[a] at the following dilution:			
	1/2	1/10	1/100	1/1,000
Hydrogen peroxide$_1$[b] (30,000)	K	K	C	T
Hydrogen peroxide$_2$[b] (30,000)	K	K	C	T
Benzalkonium chloride (40)	K	T	T	T
PABP (0.5)	C	T	T	T
Polyquaternium 1 (11)	C	T	T	T
Chlorhexidine (150)-thimerosal (50)	K	C	C	T
PYG medium	T	T	T	T

[a] K, killed protozoans; C, live cysts; T, live trophozoites.
[b] Two different brands of hydrogen peroxide solution were used.

* Corresponding author. Mailing address: Institute of Microbiology and Virology, University of Sassari, Viale San Pietro 43 B, 07100 Sassari, Italy. Phone: (079)228463. Fax: (079)212345. Electronic mail address: Biologia_Med@chss.cineca.it.

TABLE 2. Exposure times required by six commercial disinfecting solutions to kill *A. castellanii* trophozoites in vitro

Solution (concn [μg/ml]) and dilution	% Live trophozoites at the following exposure time:					
	3 min	15 min	30 min	3 h	6 h	9 h
Hydrogen peroxide$_1$[a] (30,000)						
1/2	0	0	0	0	0	0
1/10	75	55	0	0	0	0
1/100	100	80	70	90	95	100
Hydrogen peroxide$_2$[a] (30,000)						
1/2	0	0	0	0	0	0
1/10	0	0	0	0	0	0
1/100	100	80	75	90	95	100
Benzalkonium chloride (40)						
1/2	90	10	0	0	0	0
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100
PABP (0.5)						
1/2	100	100	100	100	100	100
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100
Polyquaternium 1 (11)						
1/2	100	100	100	100	100	100
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100
Chlorhexidine (150)-thimerosal (50)						
1/2	70	55	5	0	0	0
1/10	100	90	30	0	0	0
1/100	100	100	100	100	100	100
PYG medium						
1/2	100	100	100	100	100	100
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100

[a] Two brands of hydrogen peroxide were used.

TABLE 3. Exposure times required by six commercial disinfecting solutions to kill *A. castellanii* cysts in vitro

Solution (concn [μg/ml]) and dilution	% Live trophozoites at the following exposure time:					
	3 min	15 min	30 min	3 h	6 h	9 h
Hydrogen peroxide$_1$[a] (30,000)						
1/2	100	100	50	25	10	0
1/10	100	100	50	50	25	25
1/100	100	100	100	50	50	50
Hydrogen peroxide$_2$[a] (30,000)						
1/2	75	50	50	50	10	0
1/10	100	100	50	50	25	25
1/100	100	100	100	50	50	50
Benzalkonium chloride (40)						
1/2	75	50	25	25	10	10
1/10	100	100	100	50	50	50
1/100	100	100	100	100	100	100
PABP (0.5)						
1/2	100	100	100	50	50	50
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100
Polyquaternium 1 (11)						
1/2	100	100	100	100	100	100
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100
Chlorhexidine (150)-thimerosal (50)						
1/2	75	50	50	25	10	0
1/10	100	100	50	50	10	0
1/100	100	100	50	50	25	25
PYG medium						
1/2	100	100	100	100	100	100
1/10	100	100	100	100	100	100
1/100	100	100	100	100	100	100

[a] Two brands of hydrogen peroxide were used.

the controls, the degree of ameba growth, inhibition, or destruction was recorded. The minimum trophozoite amebicidal concentration (MTAC) was defined as the lowest concentration, expressed as a dilution of the commercial solutions, that resulted in the complete lysis or degeneration of trophozoites.

The exposure time required to kill trophozoites and cysts was determined as follows. Logarithmic-phase cultures of trophozoites and mature cysts were washed twice in PBS, resuspended at a concentration of 3×10^5/ml in PYG, and distributed in different 96-well plates (100 μl per well). Serial dilutions of each disinfecting solution (undiluted, 1:5, and 1:50) in PYG were performed, and 100 μl of each dilution was added to each well, giving final dilutions of 1:2, 1:10, and 1:100, respectively. Control wells received 100 μl of PYG in place of disinfecting solution. The plates were incubated at 37°C. At different times (3 min, 15 min, 30 min, 3 h, 6 h, and 9 h), the protozoa were removed from the wells, washed in PBS, and resuspended in 1 ml of PYG, and 0.2 ml of suspension was plated on NNA-*E. coli* plates. Plates were incubated at 27°C for 24 h and then examined with a light microscope at ×100 to detect viable trophozoites. All assays were performed in triplicate.

The MTACs and exposure times required to kill *A. castellanii* trophozoites and cysts are summarized in Tables 1 through 3. Data from one representative assay are shown, as results were consistent on repeated testing. Our data show that the susceptibility of *A. castellanii* to contact lens disinfecting solutions was variable depending on the stage of the organism, type and dilution of disinfecting solution, and length of exposure time. Overall, products containing hydrogen peroxide were the most active, showing the best MTAC against and the fastest inactivation of both trophozoites (3 min) and cysts (9 h). As shown in Table 2, different products containing hydrogen peroxide at the same concentration may have slightly different amebicidal effects, depending on the presence of different stabilizing ingredients. Ludwig et al. (12) reported that when hydrogen peroxide was used according to the manufacturers' recommendations (30 min of contact time), it was not effective against *A. castellanii* trophozoites and cysts.

The solution based on a chlorhexidine-thimerosal formulation showed a good amebicidal activity, with effective exposure times against both trophozoites (3 h) and cysts (9 h). Commercial solutions containing chlorhexidine were previously shown to be very effective against *A. castellanii* (8, 12, 17, 18).

The solution containing benzalkonium chloride was very effective against *A. castellanii* trophozoites at short exposure times (30 min); nevertheless, it was not so effective against cysts (complete cyst inactivation required 24 h). Hugo et al. (8) obtained similar results; conversely, Silvany et al. (17) found that solutions containing benzalkonium chloride (40 μg/ml) were very effective, killing all *A. castellanii* trophozoites and cysts after 1 h of exposure.

Solutions containing polyquaternium 1 and PABP were not effective against *A. castellanii* trophozoites and cysts. Other authors (17, 18) found similar data for polyquaternium 1. Discrepancies about PABP effectiveness have been reported in the literature. At the concentrations (0.5 to 15 μg/ml) used in commercial contact lens solutions, PABP has been found to be almost ineffective against *A. castellanii* cysts (8, 17, 18). Conversely, Larkin et al. (11) reported that the minimum cystocidal

concentration for clinical *Acanthamoeba* isolates is 1 to 4 μg/ml of PABP with exposure for 48 h. Burger et al. (2) observed that PABP concentrations ranging from 45 to 90 μg/ml are necessary to kill 99.9% of *Acanthamoeba* cysts in less than 1 h of exposure. These variations in susceptibility may in part be due to on inherent strain differences (12, 13); moreover, the protective effect of PYG medium against PABP (2) may represent another possible explanation.

It is apparent that not all commercial contact lens disinfecting solutions are alike when it comes to killing *A. castellanii*. A lack of standard methods for testing disinfecting solutions may represent one of the most critical problems when sensitivity assays are set up (13). Owing to their protected state, cysts are more resistant to killing; thus, the importance of testing disinfecting products against cysts is evident. Solutions that after dilution are still active against trophozoites and cysts are well suited for antiamebic disinfection; however, the need for adequate exposure times must be stressed.

We thank Piero Varaldo (Institute of Microbiology, University of Ancona, Italy) for his help in obtaining the samples of *A. castellanii* used in this study.

This study was supported by grants funded by MURST (40%) and RAS (60%), Italy.

REFERENCES

1. **Berger, S. T., B. J. Mondino, R. H. Hoft, P. B. Donzis, G. N. Holland, M. K. Farley, and J. E. Levenson.** 1990. Successful medical management of *Acanthamoeba* keratitis. Am. J. Ophthalmol. **110:**395–403.
2. **Burger, R. M., R. J. Franco, and K. Drlica.** 1994. Killing *Acanthamoebae* with polyaminopropyl biguanide: quantitation and kinetics. Antimicrob. Agents Chemother. **38:**886–888.
3. **Biddick, C. J., L. H. Rogers, and T. J. Brown.** 1984. Viability of pathogenic and nonpathogenic free-living amoebae in long-term storage at a range of temperatures. Appl. Environ. Microbiol. **48:**859–860.
4. **Centers for Disease Control.** 1986. *Acanthamoeba* keratitis associated with contact lenses. Morbid. Mortal. Weekly Rep. **35:**405–408.
5. **Cohen, E. J., C. J. Parlato, J. J. Arentsen, G. I. Genvert, R. C. Eagle, M. R. Wieland, and P. R. Laibson.** 1987. Medical and surgical treatment of *Acanthamoeba* keratitis. Am. J. Ophthalmol. **103:**615–625.
6. **Ficker, L.** 1988. *Acanthamoeba* keratitis—the quest for a better prognosis. Eye **2:**S37–S45.
7. **Garcia, L. S., and D. A. Bruckner.** 1993. Diagnostic medical parasitology, 2nd ed., p. 601–605. American Society for Microbiology, Washington, D.C.
8. **Hugo, E. R., W. R. McLaughlin, K. Oh, and O. H. Tuovinen.** 1991. Quantitative enumeration of Acanthamoeba for evaluation of cyst inactivation in contact lens care solutions. Invest. Ophthalmol. Vis. Sci. **32:**655–657.
9. **Kilvington, S.** 1989. Moist-heat disinfection of *Acanthamoeba* cysts. Lett. Appl. Bacteriol. **9:**187–189.
10. **Kim, B. G., P. P. McCann, and T. J. Byers.** 1987. Inhibition of multiplication in *Acanthamoeba castellanii* by specific inhibitors of ornithine decarboxylase. J. Protozool. **34:**264–266.
11. **Larkin, D. F. P., S. Kilvington, and J. K. D. Dart.** 1992. Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide. Ophthalmology **99:**185–191.
12. **Ludwig, I. H., D. M. Meisler, I. Rutherford, F. E. Bican, R. H. S. Langston, and G. S. Visvesvara.** 1986. Susceptibility of *Acanthamoeba* to soft contact lens disinfection. Invest. Ophthalmol. Vis. Sci. **27:**626–628.
13. **Meisler, M. D., and I. Rutherford.** 1991. *Acanthamoeba* and disinfection of soft contact lenses. Rev. Infect. Dis. **13:**S410–S412.
14. **Moore, M. B., J. P. McCulley, M. Luckenbach, H. Gelender, C. Newton, M. B. McDonald, and G. S. Visvesvara.** 1985. *Acanthamoeba* keratitis associated with soft contact lenses. Am. J. Ophthalmol. **100:**396–403.
15. **Moore, M. B., J. P. McCulley, C. Newton, L. M. Cobo, G. N. Foulks, D. M. O'Day, K. J. Johns, W. T. Driebe, L. A. Wilson, R. J. Epstein, and D. J. Doughman.** 1987. *Acanthamoeba* keratitis—a growing problem in soft and hard contact lens wearers. Ophthalmology **94:**1654–1661.
16. **Nagington, J., and J. E. Richards.** 1976. Chemotherapeutic compounds and *Acanthamoeba* from eye infections. J. Clin. Pathol. **29:**648–651.
17. **Silvany, R. E., J. M. Dougherty, and J. P. McCulley.** 1991. Effect of contact lens preservatives on *Acanthamoeba*. Ophthalmology **98:**854–857.
18. **Silvany, R. E., J. M. Dougherty, J. P. McCulley, T. S. Wood, R. W. Bowman, and M. B. Moore.** 1990. The effect of currently available contact lens disinfection systems on *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*. Ophthalmology **97:**286–290.
19. **Stapleton, F., D. V. Seal, and J. Dart.** 1991. Possible environmental sources of *Acanthamoeba* species that cause keratitis in contact lens wearers. Rev. Infect. Dis. **13:**S392.
20. **Visvesvara, G. S.** 1980. Free-living pathogenic amoebae, p. 704–708. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.